## REMARKS

### Specification

The disclosure is objected for containing hyperlinks on pages 20, 21, and 32. Applicants have removed the hyperlinks and made the objection moot.

### Claim Rejection under 35 USC § 112.

Claims 9-28 are rejected under 35 USC § 112 as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Office Action calls for clearer claim wording.

Applicants have amended the phrase "a first emission" and "a second emission" to "a first emission reading of the double stranded DNA dye " and "a second emission reading of the double stranded DNA dye" respectively. The phrase "emission reading of a double stranded DNA dyes" can be found in page 21, paragraph 60, of the present application.

With respect to claims 10, Applicants have cancelled claim 10 and added new claims 85-95. In newly added claims, the second emission amount for the second amplicon is the difference between the second emission reading and the third emission reading.

With respect to claim 20, Applicants amended "the emission amount" the "the first emission amount."

The word "MT" is defined in the specification. For example, the specification recites that the term"measuring temperature" or "MT'" refers to the temperature at

which an emission reading of a double stranded DNA dye is taken cycle by cycle to determine the emission amount of an amplicon. Nevertheless, Applicants have replaced the phrase "MT" with the phrase "measuring temperature" in amended claims.

The above amendments overcome the rejections of claims 9-28 under 35 USC § 112. Accordingly, Applicants respectfully requested that the rejection of claims 9-10 35 USC § 112 be reconsidered and withdrawn.

**Claim Rejections under 35 USC § 102.**

Claims 9-28 are rejected under 35 USC §102(e) as being anticipated by Wittwer et al. (US. Pat. No. 6,472, 156).

To expedite the prosecution of the present application, Applicants briefly summarize certain aspects of the present invention based on the disclosure in the specification and the disclosure in Wittwer et al. to illustrate the difference between the two.

The present specification discloses:

> One aspect of the invention is directed to methods for real-time monitoring and quantifying of multiple amplicons in a single multiplex real-time PCR reaction with the use of a double stranded DNA dye and the melting temperature discrepancy among the amplicons.
>
> *See Paragraph 8 of the present application*
>
> A double stranded DNA dye is known to fluoresce once a double stranded DNA fragment forms and fade away when the double stranded fragment unwinds into single strands or *vice versa*. Amplicons may be distinguished according to their unique melting temperatures ($T_m$s). When a PCR reaction temperature rises above an annealing and/or extension temperature and towards a denaturing temperature, the amplicon with the

lowest melting temperature denatures first, the amplicon with a higher melting temperature denatures next, and the amplicon with the highest melting temperature denatures last. The fluorescent emission of a double stranded DNA dye changes at a rate that is proportional to the rising of the reaction temperature and the incremental denaturation of amplicons. The emission difference between two emissions, one taken at a measuring temperature below the $T_m$ of an amplicon when the amplicon remains double stranded and the other taken at a measuring temperature above the $T_m$ when the double stranded DNA of the amplicon melts, reflects the emission amount of the amplicon in the double stranded status.

*See Paragraph 9 of the present application.*

The methods in the present invention substantially increase the number of amplicons to be amplified and quantitated in a single multiplex real-time PCR reaction. As known in the art, real-time quantification in multiplex PCR depends on the availability of fluorescence dyes and the discrimination of their emission wavelength. The overlap of emission interferes with the emission readings of dyes. Accordingly, so far only up to four dyes can be used for simultaneous quantification. The methods in the present invention eliminate the need for multiple dyes, since quantification depends on the melting temperature of each amplicon and the difference between a pre-$T_m$ emission and a post-$T_m$ emission emitted from a single double stranded DNA dye.

*See Paragrph 116 of the present application.*

The Wittwer et al, on the other hand, disclose:

Dyes like ethidium bromide or SYBR Green I, which preferentially bind to double-stranded DNA, can be used in any amplification and a re [sic] inexpensive. Although not sequence specific, product specificity can be increased by analysis of melting curves (Ririe et al., Anal. Biochem. 245:154-160 (1997)), or by acquiring fluorescence at a high temperature where nonspecific products have melted (Morrison et al., BioTechniques 24(6):954-958, 960, 962 (1998)). However, multiplexing by color is not possible.

Witter et al., col. 3, ll. 4-13.

The present invention provides methods and devices for analyzing sequence variations in nucleic acid samples using multiple colors. In one aspect, a method for analyzing a nucleic acid comprising multiple loci, each having two, three or more possible allelic sequences. The method

> involves combining at least a first and second pair of oligonucleotide probes with the nucleic acid sample. The first pair of probes is capable of hybridizing in proximity to each other within a segment of the nucleic acid sample comprising the first locus and the second pair is capable of hybridizing in proximity to each other within a segment of the nucleic acid sample comprising the second locus. The first member of each probe pair comprises a FRET donor and the second member comprises a FRET acceptor, the FRET acceptor of the first probe pair member having a different emission spectrum from the FRET acceptor of the second probe pair. Upon hybridization, the proximity of the first and second member of each probe pair is sufficient to allow fluorescence resonance energy transfer between the FRET donor and the FRET acceptor.
>
> Wittwer et al., col. 3, ll. 41-60.

In light of the above, Applicants note that Wittwer et al teaches methods for analyzing sequence variations in nucleic acids using multiple colors. In particular, Wittwer teaches at least two pairs of oligonucleotide probes for a nucleic acid sample, wherein the first member of each probe pair comprises a FRET donor and the second member of each probe pair comprises a FRET acceptor. In addition, the FRET acceptor of the first pair probes has a different emission spectrum form the FRET acceptor of the second pair probes. Therefore, Wittwer et al. teach differential fluorescent emission through at least two FRET acceptors with different emission spectrum or color. Wittwer et al. do not teach differential emission from the same double stranded DNA dye. Actually, Wittwer et al. states that multiplex by color is not possible for dyes binding to double-stranded DNA (Witwer et al., col. 3, ll.12-13).

Further, Applicants note that Wittwer et al. disclose that two nucleic acid probe pairs bear different Tm from each other if the two pairs have the same FRET acceptor. In other words, Wittwer et al. teach different Tm for different probes.

According to Wittwer et al. "probe" therein is meant a nucleic acid which hybridizes with specificity to a segment of a nucleic acid sample, (See Wittwer et al., col. 13, ll. 40-41) and "the nucleic acid sample is the product of one or more PCR" (See Wittwer et al., col. 14, ll. 60-64). Given further that a pair of probes is "capable of hybridizing in proximity to each other within a segment of the nucleic sample" (Wittwer et al., col. 3, ll. 48-49), the probe in Wittwer et al. is not the entire nucleic acid sample or the product of one or more PCR. Thus, though Wittwer et al. teach probe Tm, Wittwer et al does not teach Tm for PCR products. In other words, Wittwer et al. do not teach the Tm for an amplicon in the claimed invention. The amplicon of the claimed invention refers to a fragment of DNA amplified from a thermostable polymerase using a pair of primers in PCR (See para. 0084 in the present application).

In sum, since Wittwer et al do not teach differential emission from the same DNA dye, much less different Tm for PCR amplicons, Wittwer et al does not teach each and every limitations of the claimed invention. Applicants respectfully request that the rejection of claims 9-28 under 102(e) be reconsidered and withdrawn.

## CONCLUSION

The claims are now in condition for allowance. A Notice of Allowance, is respectfully requested. If Applicants can do anything more to expedite this application, Applicants request that the Examiner contact the undersigned at (310) 788-3219.

Respectfully submitted,
Perkins Coie LLP

Date: March 26, 2007

James J. Zhu, Ph.D.
Registration No. 52,396

**Correspondence Address:**
Customer No. 34055
Patent - LA
Perkins Coie LLP
P.O. Box 1208
Seattle, WA 98111-1208
Telephone: (310) 788-9900
Facsimile: (310) 788-3399